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5β-CHOLESTANE-3α,7α,12α,26-TETROL:NAD+ OXIDOREDUCTASE AND 5β-CHOLESTANE-3α,7α,12α-TRIOL-26-AL:NAD+ OXIDOREDUCTASE IN RAT LIVER

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SUMMARY

5β-Cholestane-3α,7α,12α,26-tetrol was dehydrogenated to 5β-cholestane-3α,7α,12α-triol-26-oic acid by two separate enzymes in rat liver soluble fraction, *i.e.* 5β-cholestane-3α,7α,12α,26-tetrol:NAD+ oxidoreductase and 5β-cholestane-3α,7α,12α-triol-26-al:NAD+ oxidoreductase. These two enzymes were separated by gel filtration on Sephadex G-100. Their assay methods and some properties are described.

INTRODUCTION

In the previous paper it was reported that 1 mole of 5β-cholestane-3α,7α,12α,26-tetrol (TeHC) is dehydrogenated to 5β-cholestane-3α,7α,12α-triol-26-oic acid (THCA) with concomitant reduction of 2 moles of NAD+ to NADH in rat liver soluble fraction, suggesting that two enzymes are concerned in the dehydrogenation of TeHC to THCA, namely TeHC–NAD+ oxidoreductase and 5β-cholestane-3α,7α,12α-triol-26-al (THAL)–NAD+ oxidoreductase in rat liver soluble fraction[1]. This suggestion was confirmed by separating both enzymes by gel filtration on Sephadex G-100 as previously reported[2].

In this paper the assay methods and some properties of TeHC–NAD+ oxidoreductase and THAL–NAD+ oxidoreductase, which were partially purified by $(NH_4)_2SO_4$ fractionation and by gel filtration from rat liver soluble fraction, will be reported.

MATERIALS AND METHODS

Tritium-labeled and unlabeled TeHC, THAL and THCA were prepared according to the method described by OKUDA AND DANIELSSON[3]. NAD+, NADP+, NADH and NADPH (all Grade III) were purchased from Sigma Chemical Co. (St.

Abbreviations: TeHC, 5β-cholestane-3α,7α,12α,26-tetrol; THAL, 5β-cholestane-3α,7α,12α-triol-26-al; THCA, 5β-cholestane-3α,7α,12α-triol-26-oic acid.

Louis, Mo.). Sephadex G-100 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Liver alcohol–NAD^+ oxidoreductase (EC 1.1.1.1) was assayed according to the method described by DALZIEL[4]. Liver aldehyde–NAD^+ oxidoreductase (EC 1.2.1.3) was assayed according to the method described by RACKER[5]. Absorbance at 340 nm was measured by a Hitachi spectrophotometer Model 124.

TeHC dehydrogenase, THAL reductase and THAL dehydrogenase activities were assayed according to either of the following methods. In the spectrophotometric method, a specific amount of enzyme was added to a cuvette (1-cm light path) containing 1.0 ml of buffer, 0.1 ml of 0.25 M NAD^+ and 50 μg of substrates (dissolved in 20 μl of *tert.*-butanol) in a total volume of 3.0 ml; the absorbance at 340 nm was measured by the spectrophotometer equipped with a recorder. Incubations were performed at 23°. In the chromatographic method, each enzyme activity was assayed according to the methods described before except that appropriate buffers (see RESULTS) were used[2].

The partially purified enzymes were prepared by gel filtration on Sephadex G-100 as described before[2].

RESULTS

The incubation of THAL with rat liver 100 000 × g supernatant

Fig. 1a shows a typical thin-layer chromatogram of the reaction products obtained by incubating THAL with the 100 000 × *g* supernatant from rat liver and NADH. As shown in the table the reaction products were TeHC and THCA. It seems, therefore, that a dismutation reaction occurred. Such a dismutation was also observed when THAL was incubated with the 100 000 × *g* supernatant from rat liver and NAD^+.

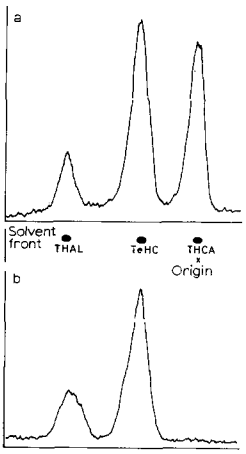


Fig. 1. Thin-layer chromatograms of the reaction products obtained by incubating THAL with either NADH and the 100 000 × *g* supernatant of rat liver (a) or Fraction 45 in Fig. 2. (b). The reaction mixture contained 0.06 μmole THAL, 0.5 μmole NADH, buffer solution (a, 50 μmoles phosphate buffer (pH 7.5); b, 50 μmoles Tris buffer (pH 7.5)) and 10 μl of enzyme solution. Incubations were conducted for 5 min at 37°. Solvent system: EA-2.

TeHC dehydrogenase, THAL reductase, THAL dehydrogenase, acetaldehyde–NAD$^+$ oxidoreductase and ethanol–NAD$^+$ oxidoreductase activities in the effluents of gel filtration

Fig. 2 shows the activities of these enzymes found in the effluents of the Sephadex G-100 column[2]. As shown in the figure, THAL dehydrogenase activity was observed in the effluent Fractions 35–39, while THAL reductase activity was found in Fractions 42–50. It is also observed that the activity profile of acetaldehyde–NAD$^+$ oxido-

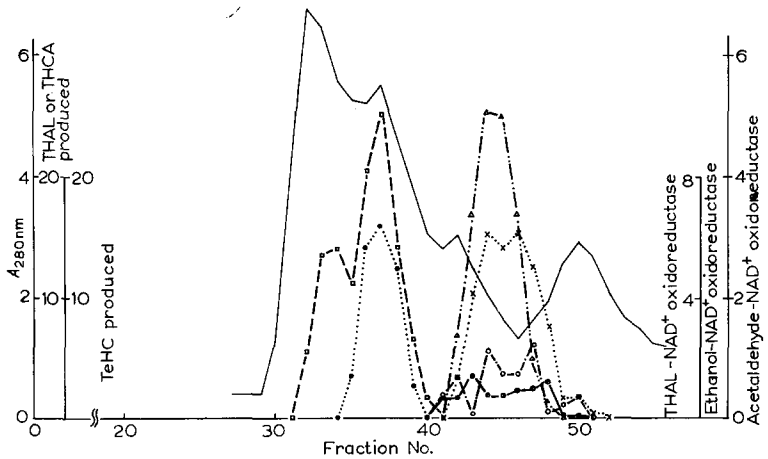


Fig. 2. TeHC dehydrogenase, THAL reductase, THAL dehydrogenase, ethanol–NAD$^+$ oxidoreductase and acetaldehyde–NAD$^+$ oxidoreductase activities in the eluate of gel filtration on Sephadex G-100 of $(NH_4)_2SO_4$ preparation (0.3–0.6 satn.) of rat liver soluble fraction. Assay methods were described in the text. ———, protein concentration ($A_{280\ nm}$); □— —□, liver acetaldehyde–NAD$^+$ oxidoreductase (units/100 μl of fraction); △—··—△, liver ethanol–NAD$^+$ oxidoreductase (absorbance increment/min per 100 μl of fraction); × ···· ×, THAL reductase (μg/5 min per 10 μl of fraction); ⊙—⊙, THAL formed and ○—·—○, THCA formed when TeHC was incubated with NAD$^+$ and an aliquot (100 μl) of fraction (μg/5 min per 100 μl of fraction); ●···●, THAL dehydrogenase (THAL–NAD$^+$ oxidoreductase) (absorbance increment/min per 50 μl of fraction).

reductase coincides with that of THAL dehydrogenase and that the activity profile of ethanol–NAD$^+$ oxidoreductase nearly coincides with that of THAL reductase. The peak for THAL dehydrogenase was found in Fraction 37 and that for THAL reductase was observed in Fraction 45. The specific activity of Fraction 37 for THAL dehydrogenase was 0.31 μmole THAL (oxidized) per 5 min per mg protein and that of Fraction 45 for THAL reductase was 3.8 μmoles THAL (reduced) per 5 min per mg protein.

In the following study an aliquot of Fraction 37 or of Fraction 45 was used as enzyme for THAL dehydrogenase or THAL reductase.

TeHC–NAD$^+$ oxidoreductase

Effect of NADH and NADPH. Fig. 3a shows the effect of the NADH concentration on THAL reductase activity. The reaction rate was determined by measuring the decrease in absorbance at 340 nm in time (1 min). The reaction rate was linear with the concentration of NADH until 10 μM. The reaction rate was very slow when NADPH was used instead of NADH as the donor (about one sixth at 80 μM).

Reaction velocity. The velocity of the THAL reduction to TeHC decreased

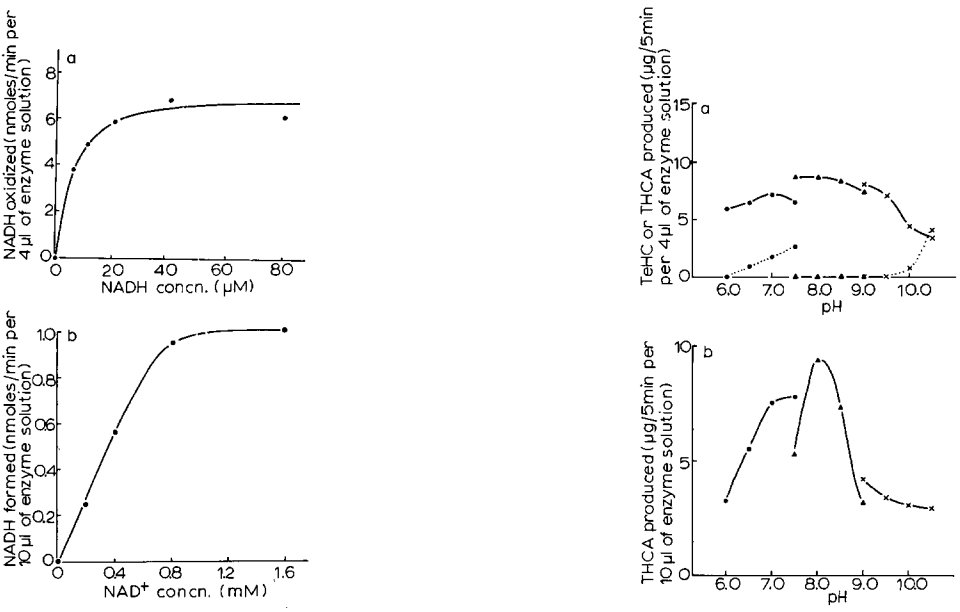


Fig. 3. Effect of coenzymes on the THAL reductase (a) and THAL dehydrogenase (b) activities. The incubation mixture contained 0.12 µmole THAL, 100 µmoles Tris buffer (pH 7.5 (a) or pH 8.0 (b)), 4 µl (a) or 20 µl (b) of enzyme solution and varying amounts of NADH or NAD^+ in a final vol. of 3.0 ml. The reaction was carried out in a cuvette at 23°.

Fig. 4. Effect of pH on the THAL reductase (a) and THAL dehydrogenase (b) activities. The incubation mixture contained 0.06 µmole THAL, 0.5 µmole NADH or 0.5 µmole NAD^+, 4 µl of Fraction 45 or 10 µl of Fraction 37 (in Fig. 2) and 50 µmoles potassium phosphate buffer (pH 6.0–7.5), 50 µmoles Tris buffer (pH 7.5–9.0) or 50 µmoles glycine buffer (pH 9.0–10.5) in a final volume of 1.5 ml. Incubations were conducted for 5 min at 37°. ———, TeHC; · · · · ·, THCA.

rapidly with time. When the reaction was assayed by measuring the decrease in absorbance at 340 nm, the reduction was linear with time only for the initial 3 min. However, when the reaction was assayed chromatographically, an incubation period of 5 min was chosen because the application of a shorter period was technically difficult. The deviation from linearity at this point was not significant judging from the results obtained spectrophotometrically.

Effect of pH. To study the effect of pH on THAL reductase, the substrate was incubated in different pH media with Sephadex G-100 eluate and NADH. As shown in Fig. 4a, a broad peak was observed in the region from pH 7.5–8.0. The formation of THCA was observed with a phosphate or glycine buffer, whereas little THCA was formed when a Tris buffer was used. Thus for the assay of THAL reductase the incubation is best carried out in the medium containing Tris buffer at pH 7.5–8.0.

Effect of enzyme concentration. Fig. 5a shows the dependence of the reaction velocity on enzyme concentration. The linearity of the enzyme concentration–reaction velocity curve is seen in the range from 2 to 10 µl of Sephadex eluate, and the deviation from linearity was observed in higher enzyme concentrations, probably due to the rapid decrease in substrate concentration.

Effect of substrate concentration. The relationship between reaction velocity and

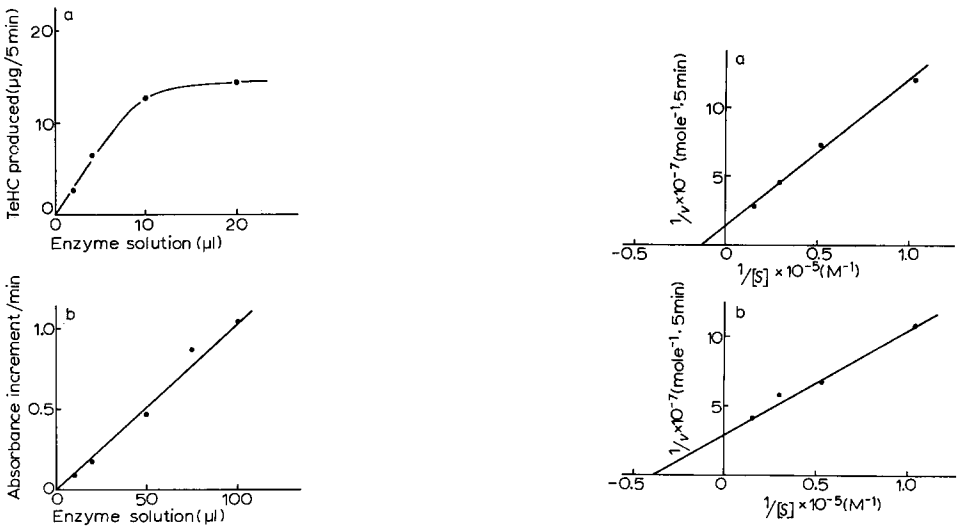


Fig. 5. Effect of enzyme concentration on the THAL reductase (a) and THAL dehydrogenase (b) activities. The assay conditions for THAL reductase were as described in Fig. 3a except that 0.5 μmole of NAD^+ and varying amounts of Fraction 45 (in Fig. 2) were used .THAL dehydrogenase was assayed spectrophotometrically as described in the text. 100 μmoles Tris buffer (pH 7.5) was used.

Fig. 6. Effect of substrate concentration on the THAL reductase (a) and THAL dehydrogenase (b) activities. Assay conditions for THAL reductase were as described in Fig. 3a except that 0.5 μmole of NAD^+ and varying amounts of THAL were used. THAL dehydrogenase was assayed as described in Fig. 5b except that 100 μmoles Tris buffer (pH 8.0) was used. Incubations were conducted for 5 min at 37°.

substrate concentration is shown in Fig. 6a. The K_m value for THAL reductase was calculated to be 6 μM.

Identification of reaction products. Fig. 1b shows a typical radiochromatogram of the product obtained by incubating THAL with Fraction 45 of the Sephadex eluate and NADH. As shown in the figure, the reaction product is only found in the area corresponding to TeHC. The labeled material in this area was eluted and recrystallized to a constant specific activity ($7.76 \cdot 10^5$ counts/min per mg) with 30 mg of cold TeHC from various solvents, it was identified as TeHC.

Stoichiometry. Stoichiometry of the enzyme reaction was studied as described in the legend of Table I. As shown in Table Ia, when THAL was incubated with an aliquot from Fraction 45 of the Sephadex G-100 eluate, using NADH as a donor, 1 mole of NADH was oxidized to NAD^+ per 1 mole of THAL reduced to TeHC. This is consistent with Reaction 1.

Reversibility. As described in the previous paper[2] and in the preceding sections, TeHC–NAD^+ oxidoreductase was assayed by measuring THAL reductase activity. However, as reported in the previous paper[2], TeHC–NAD^+ oxidoreductase could not be assayed by measuring TeHC dehydrogenase activity by our method[1]. Therefore the assay conditions for TeHC dehydrogenase were reexamined. The effect of pH on the TeHC dehydrogenase was studied in the same way as on the THAL reductase. Only a small amount of THAL (5–10%) was formed when incubation was carried out at pH above 9.0, and 10 times as much enzyme (100 μl) as used for the THAL reductase

TABLE I

STOICHIOMETRY OF REACTION

Known quantities of tritium-labeled THAL (dissolved in 10 μl of *tert.*-butanol) and NADH or NAD^+ were added to cuvettes containing 50 μmoles of Tris buffer (pH 8.0 or 7.5) and 10 μl of Fraction 45 or 20 μl of Fraction 37 of the Sephadex eluate (Fig. 2) in a final volume of 2.0 ml. Absorbance at 340 nm was recorded by a Hitachi spectrophotometer Model 124. The reaction was terminated in 15 min by adding 5 vol. of ethyl acetate and by extracting the products. Products were analyzed as described in MATERIALS AND METHODS. Oxidized or reduced NADH was calculated from the absorbance at 340 nm by taking the millimolar absorbance coefficient of NADH at 340 nm as 6.22.

Expt. No.	*pH*	*Initial NADH concn. (μM)*	*Initial NAD^+ concn. (mM)*	*Initial THAL concn. (μM)*	*NADH oxidized (μmole)*	*NAD^+ reduced (μmole)*	*TeHC formed (μmole)*	*THCA formed (μmole)*
(a) THAL reductase								
1	7.5	62.5		20.6	0.032		0.032	
2	8.0	62.5		20.6	0.029		0.028	
3	7.5	31.25		20.6	0.031		0.030	
(b) THAL dehydrogenase								
1	8.0		1	38.0		0.010		0.012
2	8.0		0.05	19.0		0.004		0.005
3	7.5		1	38.0		0.013		0.012

assay (10 μl) was used. Furthermore, when TeHC was incubated with TeHC–NAD^+ oxidoreductase, partially purified by gel filtration, larger or smaller amounts of THCA were formed. A typical pattern of TeHC dehydrogenase activity in the eluate from Sephadex G-100 was shown in Fig. 2. Although its activity was found in the fractions where THAL reductase was observed, the TeHC dehydrogenase activity was so weak that the application of this enzyme activity for assaying TeHC–NAD^+ oxidoreductase in relatively crude solution was not practical.

THAL–NAD^+ oxidoreductase

Effect of NAD^+ and $NADP^+$. Fig. 3b shows the effect of NAD^+ on the dehydrogenation of THAL to THCA by THAL–NAD^+ oxidoreductase. The reaction rate measured spectrophotometrically, was linear to 0.8 mM NAD^+. The reaction rate was very slow when $NADP^+$ was used as an acceptor (about one fifth at 0.8 mM).

Reaction velocity. The velocity of dehydrogenation of THAL to THCA was linear with time for 10–15 min judging from the results obtained by the spectrophotometric assay.

Effect of pH. To study the effect of pH on THAL dehydrogenase, the substrate was incubated in different pH media with an aliquot of Sephadex eluate Fraction 37 and NAD^+. As shown in Fig. 4b, the optimum pH seems to exist at pH 8.0, when Tris buffer (pH 8.0) was used.

Effect of enzyme concentration. Fig. 5b shows the dependence of the reaction velocity, measured spectrophotometrically, on the enzyme concentration. The linearity of the enzyme concentration–reaction velocity curve is seen in the range from 5–50 μl of Sephadex eluate from Fraction 37.

Effect of substrate concentration. The relation between reaction velocity and

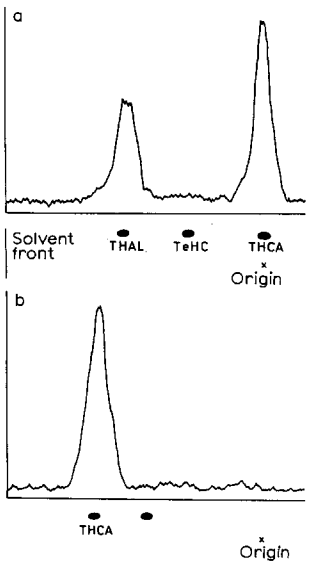


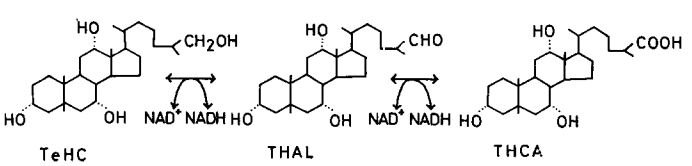
Fig. 7. Thin-layer chromatogram in solvent system EA-2 (a) of the reaction products of THAL by the partially purified THAL–NAD^+ oxidoreductase (Fraction 37 in Fig. 2) fortified with NAD^+ and the rechromatogram of the acidic material in solvent system S-7 (b). Incubations were conducted as described in the text.

substrate concentration is seen in Fig. 6b. The K_m value for THAL dehydrogenase was calculated to be 22 μM.

Identification of products of reaction. Fig. 7a shows a typical radiochromatogram of the products obtained by incubating THAL with Sephadex G-100 eluate Fraction 37 and NAD^+. As shown in the figure, the reaction product was only found in the origin; because the acidic material does not move from the origin in this solvent system (EA-2 (ref. 6)), the labeled material in the origin was eluted and rechromatographed on a thin-layer plate using phase system S-7 (ref. 7) (Fig. 9b). As clearly shown in the figure, radioactivity was observed in the area corresponding to THCA. The labeled material in this area was eluted and recrystallized to a constant specific activity ($4.65 \cdot 10^4$ counts/min per mg) with 30 mg of cold THCA from various solvents, it was identified as THCA.

Stoichiometry. Stoichiometry of the enzyme reaction was studied as described in the legend of Table I. As shown in Table Ib, when THAL was incubated with an aliquot of Sephadex eluate Fraction 37 and NAD^+, 1 mole of NAD^+ was reduced to NADH per 1 mole of THAL oxidized to THCA. This is consistent with Reaction 1.

Reversibility of enzyme reaction. Incubation of THCA with an aliquot of Fraction 37 of Sephadex G-100 and NADH at different pH's did not result in any detectable formation of THAL.



DISCUSSION

Although THAL is not stable for long periods, it can be stored for a few days without serious deterioration under N_2. According to the control incubation with boiled enzyme, it was found that THAL was not autooxidized appreciably during incubation, extraction and following analysis of the products.

In the course of this study, some results obtained with the incubations conducted in the mixture containing 10 μl of methanol (used for solvent for THAL) in a total volume of 1.5 ml were compared with those obtained with the incubations conducted in the mixture containing 10 μl of *tert.*-butanol (used for solvent for THAL) or with those obtained with the incubations conducted in the mixture containing no organic solvent. It was evaporated by blowing N_2 after introduction of THAL solution into test tubes before introducing the reagents. It was found that there is no essential difference among them.

As previously reported, when TeHC is incubated with rat liver soluble fraction, TeHC is rapidly dehydrogenated to THCA without appreciable accumulation of THAL[1]. On the other hand, when THAL was incubated with rat liver soluble fraction to assay TeHC–NAD^+ oxidoreductase by reverse reaction, dismutation reaction was inevitable as shown in Fig. 1a. According to RACKER[8], such a dismutation is actually a coenzyme-linked dismutation catalyzed by two dehydrogenases which catalyze successive steps in the oxidation of ethanol. Therefore, it was impossible to measure only the activity of TeHC–NAD^+ oxidoreductase or THAL–NAD^+ oxidoreductase in the rat liver soluble fraction. Unless only the activity of these enzymes was measured, it was impossible to investigate the properties of the individual enzyme. By separating the two enzymes, the problem was eventually solved, and the properties of these enzymes can now be described.

Although both assay methods for THAL–NAD^+ oxidoreductase and TeHC–NAD^+ oxidoreductase were used for the purified enzyme, the spectrophotometric method could not be used for a crude preparation containing both enzymes because, in this case, coenzyme-linked dismutation occurred and because the absorbance at 340 nm did not change appreciably. On the other hand, the chromatographic method revealed the dismutation and suggested the existence of both oxidoreductases. For this reason, although the spectrophotometric method is more rapid and accurate than the chromatographic method, it is necessary to correlate the results obtained when relatively crude preparations are used.

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